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Title: THE JOURNAL OF IMMUNOLOGY.

Volume: 137 Issues: 1-6

Date: JULY 1 - SEPTEMBER 15, 1986

UMI Number: 79.00

Note: REEL NO: 3 OF 4

Includes:
INDEX VOLUME 137 (1986)
CONTENTS VOLUME 137 (1986)

UMI
300 North Zeeb Road
Ann Arbor, MI 48103

0022-1767/86/1374-1270$02.00/0
The Journal of Immunology

Vol. 137, 1270–1274, No. 4, August 15, 1986
Printed in U.S.A.

A HUMAN INTERCELLULAR ADHESION MOLECULE (ICAM-1) DISTINCT FROM LFA-1[1]

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Homotypic adhesion by phorbol ester-stimulated lymphocytes requires LFA-1 and Mg^{+2} and does not involve like-like interactions between LFA-1 molecules on adjacent cells. The latter finding suggested that a second molecule, distinct from LFA-1, also participates in LFA-1-dependent adhesion. The identification of such a molecule was the object of this investigation. After immunization with LFA-1-deficient EBV-transformed lymphoblastoid cells, a MAb was obtained that inhibits phorbol ester-stimulated aggregation of LFA-1$^+$ EBV lines. This MAb defines a novel cell surface molecule, which is designated intercellular adhesion molecule 1 (ICAM-1). ICAM-1 is distinct from LFA-1 in both cell distribution and structure. In SDS-PAGE, ICAM-1 isolated from JY cells is a single chain of M_r = 90,000. As shown by MAb inhibition, ICAM-1 participates in phorbol ester-stimulated adhesion reactions of B lymphocyte and myeloid cell lines and T lymphocyte blasts. However, aggregation of one T lymphocyte cell line (SKW-3) was inhibited by LFA-1 but not ICAM-1 MAb. It is proposed that ICAM-1 may be a ligand in many, but not all, LFA-1-dependent adhesion reactions.

A number of "accessory" molecules that cooperate with specific receptors and play an important role in the cell interactions of the immune system have recently been defined (1, 2). One of these is the lymphocyte function-associated 1 (LFA-1)[3] antigen, which cooperates with the antigen receptor in T cell-mediated killing and helper cell responses, and with the Fc receptor in antibody-dependent cell-mediated killing (1, 2). Recently, LFA-1 was shown to mediate adhesion of activated lymphocytes in the absence of specific receptor-ligand interactions (3). Lymphocyte activation with phorbol esters induces an increase in adhesiveness that may be measured by the formation of cell clusters. Phorbol ester-stimulated homotypic adhesion by peripheral blood lymphocytes, Epstein Barr virus (EBV)-transformed B cell lines, a T cell line, and a myeloid cell line is inhibited by LFA-1 m noclonal antibodies (MAb). Furthermore, EBV-transformed cell lines derived from LFA-1-deficient patients fail to self-aggregate, in contrast to cell lines established from healthy relatives or healthy controls. Although LFA-1$^-$ cells do not self-aggregate, they can co-aggregate with LFA-1$^+$ cells. This ruled out the possibility of like-like recognition in which LFA-1 on one cell would bind to LFA-1 on another cell. Two possibilities remain: that LFA-1 mediates adhesion by binding to a distinct ligand, or that LFA-1 regulates adhesion but does not directly participate in receptor-ligand interactions. In either case, at least one additional leukocyte cell surface molecule is predicted to be of importance in adhesion reacti ns. Therefore, to detect such a molecule, MAb were elicited by immunization with cells genetically deficient in LFA-1 and screened for their ability to inhibit aggregation of LFA-1$^+$ cells. We report here the use of MAb to identify and characterize a molecule distinct from LFA-1, which is involved in phorbol ester-stimulated homotypic adhesion.

MATERIALS AND METHODS

Preparation of MAb. BALB/c mice (three each) were immunized i.p. with EBV-transformed peripheral blood mononuclear cells from LFA-1-deficient patient 2 (4) (10^7 cells in 1 ml RPMI 1640 medium) on day 45, day 29, and day 4 before fusion. On day 3 before fusion, the mice were given an additional 10^7 cells in 0.15 ml medium i.v. Spleen cells were fused with P3X63Ag8.653 myeloma cells at a ratio of 4:1 according to the protocol of Galfre et al. (5). Hybridomas were aliquoted into 96-well microtiter plates. The hybridoma supernatants were screened for inhibition of aggregation, and one inhibitory hybridoma was cloned and subcloned by limiting dilution. The subclone used here was designated RR1/1.1.1 or RR1/1 for brevity. The TS1/22 MAb specific for the LFA-1 α subunit (6), TS1/18 MAb specific for the LFA-1 β subunit (6), TS2/9 LFA-3 MAb (7), and W6/32 framework HLA-A,B MAb (8) have been described. P3X63 IgI myeloma supernatant was used as control.

Aggregation assay. A qualitative aggregation assay was carried out as described (3). Briefly, 2 × 10^5 cells in 100 µl RPMI 1640 medium with 10% fetal bovine serum were added to a flat-bottomed 96-well microtest plate. The appropriate hybridoma supernatant (50 µl) and phorbol 12-myristate 13-acetate (PMA) (50 µl of 200 ng/ml) were then added. Cells were incubated for 2 to 18 hr at 37°C and viewed with an inverted microscope for scoring aggregation. Because different cell lines aggregate at different rates, the length of the aggregation assay was varied to maximize PMA-induced aggregation. Scores ranged from 0 to 5+, where 0 indicates that essentially no cells were in clusters; 1+ indicates less than 10% of the cells were in aggregates; 2+ indicates that 10 to 50% of the cells were aggregated; 3+ indicates that 50 to 100% of the cells were in small, loose clusters; 4+ indicates that up to 100% of the cells were aggregated in larger clusters; and 5+ indicates that 100% of the cells were in large, very compact aggregates. For reference, the scores in Figure 1 are as follows: *A* and *D*, 3+; *B*, *C*, and *E*, 0.

To quantitate aggregation, 4 × 10^5 PHA-blasts (4) in 100 µl RPMI 1640 plus 10% fetal bovine serum were added to 100 µl of hybridoma supernatant in 12 × 75-mm plastic tubes. Where indicated, the cells were either pretreated with 50 g/ml PMA at 37°C for 4 hr before

Received for publication April 26, 1986.
Accepted for publication May 22, 1986.

[1] This work was supported by Council for Tobacco Research Grant 1307 and Grants AI05877 and CA31798 from the National Institutes of Health.
[2] Supported by an American Cancer Society faculty award.
[3] Abbreviations used in this paper: LFA-1, lymphocyte function-associated antigen-1; PMA, phorbol-12-myristate acetate; MAb, monoclonal antibody; ICAM-1, intercellular adhesion molecule-1.

the addition of MAb or were treated with PMA at the time of MAb addition. The tubes were incubated at 37°C on a gyratory shaker at 75 to 175 rpm as indicated. At 1 or 2 hr after the addition of MAb, the tubes were gently vortexed for 3 sec, and the number of nonaggregated cells was determined by counting 10 μl of the suspension in a hemacytometer. The number of total cells was similarly determined after the addition of 10 mM EDTA, and incubation at 4°C for 30 min, followed by vigorous vortexing. Percent aggregation was determined by the following equation:

$$\% \text{ Aggregation} = \left(1 - \frac{\text{number of free cells}}{\text{number of total cells}}\right) \times 100$$

Immunofluorescence. Cells were stained by using an indirect immunofluorescent technique, subjected to flow cytometry, and fluorescence intensity was quantitated according to Kurzinger et al. (9). Affinity-purified fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Zymed) was used as the second antibody.

Immunoprecipitation and SDS-PAGE (10). JY cells were lysed at 5×10^7 cells/ml in 1% Triton X-100, 0.14 M NaCl, 10 mM Tris, pH 8.0, with freshly added 1 mM phenylmethylsulfonylfluoride, 0.2 trypsin inhibitor units/ml aprotinin (lysis buffer) for 20 min at 4°C. Lysates were centrifuged at 10,000 × G for 10 min and precleared with 50 μl of a 50% suspension of CNBr-activated, glycine-quenched Sepharose CL-4B for 1 hr at 4°C. One milliliter of lysate was immunoprecipitated with 20 μl of a 50% suspension of MAb coupled to Sepharose CL-4B (1 mg/ml) overnight at 4°C (4). MAb-Sepharose was prepared by using CNBr activation of Sepharose CL-4B in carbonate buffer (11). Washed immunoprecipitates were subjected to SDS-PAGE and silver staining (12).

RESULTS

Hybridomas were produced from mice immunized with LFA-1-deficient EBV-transformed B lymphoblastoid cells. Supernatants from over 600 hybridoma wells were screened for inhibition of PMA-stimulated JY cell aggregation. A single culture supernatant inhibited aggregation, and inhibitory clones and subclones from this culture were selected. The MAb secreted by the subcloned hybridoma designated RR1/1 consistently inhibited PMA-stimulated aggregation of the LFA-1-positive EBV cell line JY (Fig. 1*E*). The RR1/1 MAb inhibits aggregation equivalently, or slightly less than MAb to the LFA-1 α or β subunits (Fig. 1*B* and *C*). In contrast, control MAb against HLA, which is abundantly expressed on JY (Table I), did not inhibit aggregation (Fig. 1*D*). We have designated the antigen defined by RR1/1 as intercellular adhesion molecule-1 (ICAM-1).

It was important to determine whether ICAM-1 was distinct from LFA-1, particularly since patient cells, although negative for cell surface LFA-1, synthesize an intracellular LFA-1 α-chain precursor, which might have been immunogenic. Immunofluorescence flow cytometry sh wed that the ICAM-1 MAb reacted equally well with EBV-transformed cell lines from both healthy LFA-1$^+$ and LFA-1-deficient individuals (Table I).

Immunoprecipitation of ICAM-1 from JY cells further showed that it was distinct from LFA-1 (Fig. 2). ICAM-1 precipitates formed with MAb-Sepharose were subjected t SDS-PAGE and visualized by silver staining. ICAM-1 migrates as a broad band of M_r = 90,000 under both reducing (Fig. 2*A*, lane 1) and nonreducing conditions (Fig. 2*B*, lane 1). Bands of M_r = 50,000 and 25,000 corresponded to Ig H and L chains from the MAb Sepharose (Fig. 2*A*, lane 3). Variable amounts of other bands in the 50,000 to 25,000 M_r range were also observed, but were not seen in precipitates from hairy leukemia cells, which yielded only the 90,000 M_r material (not shown). The M_r = 177,000 α subunit and M_r = 95,000 β subunit of LFA-1 (13, 14) migrated distinctly from ICAM-1 under both reducing (Fig. 2*A*, lane 2) and nonreducing (Fig. 2*B*, lane 2) conditions.

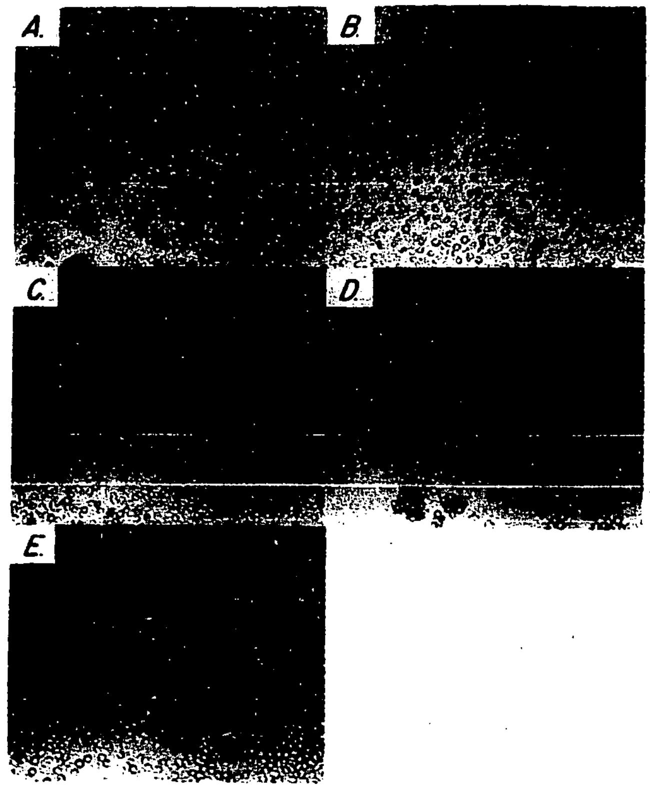

Figure 1. JY cell aggregation is inhibited by MAb to ICAM-1. Photomicrographs of JY cells aggregating in the presence of PMA with *A*, no monoclonal antibody; *B*, anti-LFA-1 β-chain (TS1/18); *C*, anti-LFA-1 α-chain (TS1/22); *D*, anti-HLA framework (W6/32); and *E*, anti-ICAM-1 (RR1/1).

TABLE I
Expression of LFA-1 and ICAM-1 on EBV-transformed B lymphoblastoid cells from healthy and LFA-1-deficient individuals[a]

MAb	Specificity	Specific Linear Fluorescence[b]	
		Healthy	Patient 2
TS1/18	LFA-1 β chain	24	1
RR1/1	ICAM-1	35	63
W6/32	HLA	257	295
TS2/9	LFA-3	58	65

[a] EBV-transformed cells established in parallel from patient 2 or a healthy control were treated with 50 ng/ml of PMA for 2 hr at 37°C before staining.
[b] After subtraction of background fluorescence (three for healthy and five for patient 2 EBV lines).

Of many MAb tested, only ICAM-1 and LFA-1 MAb inhibited phorbol ester-stimulated leukocyte aggregation. Anti-HLA-A,B MAb (Table II) and anti-LFA-3 MAb (3) did not inhibit. This demonstrates the specificity of inhibition, since LFA-3 and HLA-A,B antigens are present in higher density on EBV-transformed cell lines than are ICAM-1 or LFA-1 (Table I). Parallel studies with 115 different MAb in a leukocyte workshop showed that only those to LFA-1 inhibited aggregation, further demonstrating specificity (3). Thus, only anti-ICAM-1 MAb or LFA-1 MAb inhibit aggregation.

We examined the ability of ICAM-1 MAb t inhibit aggregation of several different types of cell lines. ICAM-1 MAb consistently inhibited PMA-stimulated aggregation of the JY B lymphoblastoid cell line (Fig. 1 and Table II) and three other EBV-transformed B lymphoblastoid cell lines in many different expen .ents (data not shown).

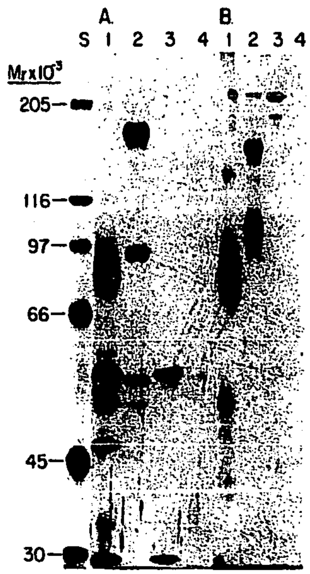

Figure 2. Immunoprecipitation of ICAM-1 and LFA-1 from JY cells. Triton X-100 lysates of JY cells (lanes 1 and 2) or control lysis buffer (lanes 3 and 4) were immunoprecipitated with ICAM-1 MAb-Sepharose (lanes 1 and 3) or LFA-1 MAb-Sepharose (lanes 2 and 4). After elution of proteins with SDS sample buffer at 100°C, the samples were divided in half and subjected to SDS-8% PAGE under reducing (*A*) and nonreducing conditions (*B*). Proteins were visualized by silver staining. Molecular weight standards were run in lane S.

TABLE II

Inhibition of PMA-induced aggregation with LFA-1 and ICAM-1 MAb[a]

Cell Type	Control	Aggregation		
		LFA-1[b]	ICAM-1	HLA[c]
JY	5+	0	1+	5+
U937	3+	0	1+	4+
SKW-3	3+	0	3+	4+

[a] Fresh JY cells, fresh SKW-3 cells, or U937 cells that had been precultured for 3 days in 2 ng/ml PMA were stimulated with 50 ng/ml PMA for 2 hr, and aggregation was scored visually.
[b] TS1/18 anti-β.
[c] W6/32.

TABLE III

Effect of PMA on expression of membrane LFA-1 and ICAM-1

Cell Type	PMA Treatment[a]	Specific Fluorescence Intensity	
		LFA-1[b]	ICAM-1
JY	—	56	55
	50 ng/ml, 4 hr	56	63
SKW-3	—	85	1.8
	50 ng/ml, 4 hr	72	1.6
U937	—	17	6.9
	2 ng/ml, 3 days	41	120

[a] TS1/22 anti-LFA-1 α-chain MAb.
[b] Cells were treated with PMA under conditions that yielded optimum aggregation for each cell type.

The myelomonocytic cell line U937 did not aggregate in a 2-hr assay unless maturation along the monocytic pathway had previously been induced by culture with PMA f r 3 days. U937 cells cultured with PMA for 3 days were dispersed into single cell suspensi ns by vortexing and were tested for aggregation in a 2-hr assay (Table II). ICAM-1 and LFA-1 MAb, but not HLA-A,B MAb, inhibited differentiated U937 cell aggregation in repeated experiments. However, aggregation of the SKW-3 T cell lymphoma line was found to be inhibited by LFA-1 but not by ICAM-1 MAb (Table II).

ICAM-1 and LFA-1 expression by JY, SKW-3, and U937

TABLE IV

Inhibition of PMA-stimulated PHA-lymphoblast aggregation by RR1/1 MAb[a]

Expt.	PMA	MAb	% Aggregation	% Inhibition[b]
1[c]	−	Control	9	—
	+	Control	51	0
	+	HLA-A,B	58	−14[f]
	+	LFA-1 α	31	39
	+	ICAM-1	31	39
2[d]	−	Control	10	—
	+	Control	78	0
	+	LFA-1 β	17	78
	+	ICAM-1	50	36
3[e]	−	—	7	
	+	Control	70	
	+	HLA-A,B	80	−14
	+	LFA-3	83	−19
	+	LFA-1 α	2	97
	+	LFA-1 β	3	96
	+	ICAM-1	34	51

[a] Aggregation of PHA-induced lymphoblasts stimulated with 50 ng/ml PMA was quantitated indirectly by microscopically counting the number of nonaggregated cells as described in *Materials and Methods*.
[b] Percent inhibition relative to cells treated with PMA and X63 MAb.
[c] Aggregation was measured 1 hr after the simultaneous addition of MAb and PMA. Cells were shaken at 175 rpm.
[d] Aggregation was measured at 1 hr after the simultaneous addition of MAb and PMA. Cells were pelleted at 200 × G for 1 min, incubated at 37°C for 15 min, gently resuspended, and shaken for 45 min at 100 rpm.
[e] Cells were pretreated with PMA for 4 hr at 37°C. After MAb were added, the tubes were incubated at 37°C stationary for 20 min and shaken at 75 rpm for 100 min.
[f] A negative number indicates percent enhancement of aggregation.

cells and the effect of PMA treatment were determined by immunofluorescence flow cytometry (Table III). JY cells expressed equivalent amounts of LFA-1 and ICAM-1, whereas SKW-3 expressed relatively high amounts of LFA-1 and low amounts of ICAM-1. Stimulation with PMA under conditions optimal for induction of aggregation had no effect on ICAM-1 or LFA-1 expression by JY or SKW-3 cells. When U937 cells were stimulated to differentiate into monocyte/macrophage-like cells by cultivation in PMA for 3 days, the amount of LFA-1 was increased less than threefold, whereas ICAM-1 was dramatically increased 17-fold. U937 cells cultured for 3 days with PMA aggregated strongly; short-term stimulated U937 cells did not (3). This increased aggregation correlates with induction of ICAM-1 expression.

To examine the importance of ICAM-1 in adhesion by activated T cells, we tested T cell blast aggregation. T cells stimulated 4 days with PHA were thoroughly washed, then cultured for 6 days in IL 2 before assay. PHA is internalized during this 6-day culture and does not contribute to the aggregation assay. In three different assays with different T cell blast preparations, ICAM-1 MAb consistently inhibited aggregation (Table IV). LFA-1 MAb was consistently more inhibitory, whereas HLA-A,B and LFA-3 MAb were without effect.

DISCUSSION

A novel antigen has been defined that is functionally important in PMA-stimulated homotypic aggregation by leukocytes. This molecul has been designated intercellular adhesion molecule-1, or ICAM-1. Previously, only the LFA-1 molecule has been shown to be involved in leukocyte homotypic adhesion (3, 15, 16). LFA-1[+] cells have been shown to adhere to LFA-1[−] cells in an LFA-1-dependent reaction (3). Therefore, we postulated the existence of additional adhesion molecules. MAb were elicited t LFA-1[−] cells and screened for inhibition of PMA-

stimulated aggregation of LFA-1$^+$ cells. This resulted in the identification of ICAM-1.

ICAM-1 is a cell surface molecule of 90,000 M_r in SDS-PAGE under both reducing and nonreducing conditions. ICAM-1 is distinct in M_r from LFA-1, which has two chains of M_r = 177,000 and 95,000. ICAM-1 can be further distinguished from LFA-1 by its presence on genetically LFA-1-deficient cells. The ICAM-1 molecule is also distinct in M_r from the accessory molecules LFA-2 (CD2) and LFA-3, which are M_r = 49,000 and M_r = 60,000, respectively (7). LFA-3 has been proposed as a possible LFA-1 ligand (17). However, MAb to LFA-3 do not inhibit PMA-stimulated leukocyte aggregation, in contrast to LFA-1 and ICAM-1 MAb.

Inhibition of cell adhesion by MAb to ICAM-1 appears to be highly specific, since MAb binding to a higher number of cell surface sites, such as HLA MAb, do not inhibit aggregation. We have tested a large number of MAb to cell surface antigens, and only those to LFA-1 or ICAM-1 inhibit PMA-stimulated leukocyte homotypic aggregation.

To examine the means by which phorbol esters stimulate leukocyte aggregation, we tested the effects of PMA on expression of ICAM-1 and LFA-1. Rapid stimulation of B lymphoblastoid cell aggregation by phorbol esters was not accompanied by any quantitative change in ICAM-1 or LFA-1 expression. Other changes thus appear important in regulating ICAM-1- and LFA-1-dependent adherence. ICAM-1 expression may, however, play a role in regulating adhesion of a monocytic cell line. During culture for 3 d with PMA, U937 cells mature along the monocytic pathway (18) and coordinately increase ICAM-1 expression 17-fold. ICAM-1 modulation directly correlates with acquisition of competence for aggregation by U937 cells.

In further studies, we found that ICAM-1 is a widely distributed antigen and that its expression on fibroblasts (19) and endothelial cells (20) is rapidly increased by IL 1 and several other cytokines. ICAM-1 may be important in regulating adhesiveness in inflammation. Adherence of T cell blasts or SKW-3 cells to fibroblasts is increased by IL 1 pretreatment of fibroblasts, correlating with ICAM-1 up-modulation. This adherence is inhibited by LFA-1 MAb bound to the T cells, or by ICAM-1 MAb bound to fibroblasts (19). This is consistent with the hypothesis that ICAM-1 is a ligand for LFA-1. The findings in the present study that ICAM-1 MAb inhibit LFA-1-dependent adhesion by B lymphoblastoid lines, T cell blasts, and a monocytic cell line are also consistent with the hypothesis that ICAM-1 is a ligand for LFA-1. However, the ability of LFA-1 MAb but not ICAM-1 MAb to inhibit SKW-3 cell homotypic aggregation argues against the notion that ICAM-1 is a ligand on SKW-3 cells. It remains possible that ICAM-1 is a ligand for LFA-1 in many types of cellular interactions, and that a different molecule acts as a ligand for LFA-1 in SKW-3 cell homotypic aggregation. ICAM-1 may be a member of a family of ligand molecules, only some of which express the epitope recognized by the RR1/1 MAb.

The studies reported here define a novel leukocyte cell surface molecule and demonstrate its importance in phorbol ester-stimulated homotypic aggregation of a number of leukocyte cell lines and T cell blasts. The mechanism of action at the molecular level of ICAM-1, and of leukocyte adherence molecules in general, requires further investigation. They may directly participate in receptor-ligand interactions or may regulate adhesion indirectly. Four leukocyte adherence or "accessory molecules" have now been identified: LFA-1, LFA-2 (CD2), LFA-3 (7, 21), and ICAM-1. The identification of ICAM-1 in these studies will allow further testing at the molecular level of the hypothesis that LFA-1 interacts with a ligand, and of the molecular basis of leukocyte adhesion.

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